AD_____

Award Number: W81XWH-11-1-0172

TITLE: Oxidative stress increases the blood brain barrier permeability resulting in increased incidence of brain metastasis in BRCA mutation carriers.

PRINCIPAL INVESTIGATOR: Hava Avraham, Ph.D.

CONTRACTING ORGANIZATION: Beth Israel Deaconess Medical Center
Boston, MA 02215

REPORT DATE: February 2013

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
February 2013	Annual	24 January 2012 – 23 January 2013

4. TITLE AND SUBTITLE

Oxidative stress increases the blood brain barrier permeability resulting in increased incidence of brain metastasis in BRCA mutation carriers

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-11-1-0172

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Hava Avraham, Ph.D.

E-Mail: havraham@bidmc.harvard.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Beth Israel Deaconess Medical Center
Boston, MA 02215

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

BRCA1 is a multifunctional tumor suppressive protein. Knockout of WT BRCA1 in breast cancer cells resulted in an increase in cell proliferation, anchorage-independent growth, cell migration, invasion and a loss of p21/Waf1 and P27Kip1 expression. Further, in BRCA1 knocked-down cells, the expression of survivin was significantly up-regulated with a decrease in cellular sensitivity to paclitaxel. Cells that harbor endogenous mutant or defective BRCA1 (such as MDA-MB-436 and HCC1937) were highly proliferative and expressed a relatively low levels of p21/Waf1 and p27Kip1 and high level of survivin and were resistant to paclitaxel. Thus, mutated BRCA1 or loss of WT BRCA1 up-regulates the malignant cell behavior. However, it is still not clear how tumor cells expressing mutant BRCA1 have enhance tumorogenicity in vivo.

15. SUBJECT TERMS
BRCA1, Knockout Mice, Breast Cancer Cells, BRCA1

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	15	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

INTRODUCTION

BRCA1 is a multifunctional tumor suppressive protein. Knockout of WT BRCA1 in breast cancer cells resulted in an increase in cell proliferation, anchorage-independent growth, cell migration, invasion and a loss of p21/Waf1 and P27Kip1 expression. Further, in BRCA1 knocked-down cells, the expression of survivin was significantly up-regulated with a decrease in cellular sensitivity to paclitaxel. Cells that harbor endogenous mutant or defective BRCA1 (such as MDA-MB-436 and HCC1937) were highly proliferative and expressed a relatively low levels of p21/Waf1 and p27Kip1 and high level of survivin and were resistant to paclitaxel. Thus, mutated BRCA1 or loss of WT BRCA1 up-regulates the malignant cell behavior. However, it is still not clear how tumor cells expressing mutant BRCA1 have enhance tumorogenicity in vivo.

BODY

Hypothesis

We hypothesize that adhesion of BRCA1 mutated cells to endothelial cells activates several distinct signaling pathways to induce MMP gene expression and increased ROS levels. We hypothesize that oxidative stress induced by adhesion of cells with mutated BRCA1 to HBMEC results in alterations in the integrity of the BBB and changes of the tight-junctions, leading to transmigration of tumor cells across the BBB and colonization of these cells in the brain forming breast cancer metastasis in the brain.

Specific Aims:

1) Elucidate the molecular mechanisms and signaling pathways by which adhesion of breast cancer cells expressing mutated BRCA1, as compared to breast cancer cells expressing WT-BRCA1, induces ROS production in human brain microvascular endothelial cells.

2) Examine the effects of oxidative stress on tight junction expression (ZO-1, ZO-2, occludin, and claudin-5), permeability and integrity of the brain endothelium using in vitro and In vivo models.

3) Determine the protective effects of PARP inhibitors and/or selenium in preventing BBB-induced damage by oxidative stress, and in inhibiting breast cancer metastasis to the brain. Further, since selenium has anti-cancer properties that are linked with protection against oxidative stress and Poly (ADP-ribose) polymerase (PARP) inhibitors have shown activity against BRCA1 and BRCA2 deficient cancers, we will therefore analyze their therapeutic potential to inhibit damage to the BBB and transmigration of tumor cells across the BBB.

Summary of Results:

Year 1

We have shown that adhesion of breast cancer cells expressing mutant BRCA1 (HCC 1937) to human brain microvascular endothelial cells (HBMECs) was significantly higher and was mediated via ROS production of tumor cells. Further, increased migration of HCC1937cells was observed as compared to HCC1937cells stably expressing WT BRCA-1. HCC1937cells induced changes in permeability of HBMECs which were more pronounced in the presence of HCC1937as compared to HCC 1937/WT-BRCA1 and these changes were inhibited by both ROS and PARP inhibitors.

Year 2

Aims: 2) Examine the effects of oxidative stress on HBMEC-tight junction expression (ZO-1 and Claudin-5) in vitro and in vivo.

3) Determine the protective effects of PARP and ROS inhibitors, as well as COX-2 inhibitors in preventing BBB induced change and breast tumor colonization in brain.

Results:

Since the BBB function is maintained by the presence of a continuous tight junction (TJ) complex, we investigated the effects of HCC1937 cells expressing mutant BRCA1, as compared to HCC1937/WT-BRCA1 expressing WT BRCA1 in HBMECs 3D cocultures of human astrocytes together with HBMEC system, on HBMEC-TJ integrity. While HBMECs expressed a linear and continuous distribution at cell-cell boundaries with normal ZO-1 and claudin-5 distribution (Fig. 1A), the cocultures of HCC1937 expressing mutant BRCA1 resulted in significantly damaged and less continuous, non-linear appearance in HBMECs or completely missing at several cell-cell boundaries (Fig. 1C). Interestingly, HCC1937/BRCA1 was capable of maintaining some TJ structures in HBMECs (Fig. 1B), and the damage of BMEC-TJs was less severe as compared to that of HCC1937.

The addition of ROS inhibitor or COX-2 inhibitor directly to HBMEC cultures was toxic and caused apoptosis of HBMEC at 50nM (Fig. 2A) or at 25nM (data not shown). In cocultures of HBMECs and HCC1937 (Fig. 3B) and HCC1937/WT-BRCA1 (Fig. 3A), ROS inhibitor by itself was unable to prevent damage of TJ structures in HBMECs (Fig. 3). However, ROS inhibitor showed some protective effects on HBMEC-TJs ZO-1 and cluadin-5, as compared to cocultures with HCC1937 cells. Further, PARP inhibitor at 40μM was also toxic to HBMEC cocultures (Fig. 2B)

Since we observed some protective effect with HCC1937/WT-BRCA1, we examined the expression of TJ structures (ZO-1 and claudin-5) in the presence of HCC1937//WT-BRCA1 in more details. The presence of TJs ZO-1 and claudin-5 on HBMECs was less damaged (Fig. 4B) as compared to control HBMECs (Fig. 4A). Interestingly, these changes in TJs were minimized in the presence of COX-2 inhibitor (Fig. 4C). These data strongly suggest that mutated BRCA1 expressing tumor cells are more aggressive in causing damage to BMECs and the BBB. Interestingly, we observed that HCC1937 cells express high level of COX-2 by RT-PCR (Fig. 5A) and western blotting (Fig. 5B), whie HCC1937/WT-BRCA1 had no expression of COX-2 (Fig. 5B). Thus, WT-BRCA1 inhibits COX-2 expression in tumor cells.

Next, we performed in vivo studies on the effects of MT-BRCA1 expressing tumor cells on the BBB integrity, TJ complexes in BMECs, and mammary tumor colonization in brain. Mice were injected with GFP-HCC-1937 or with GFP-HCC-1937/WT BRCA1 to the carotid artery (10^6 cells). After 7 weeks, the mice were euthanized and the BBB permeability in the brain of these mice was analyzed. As shown in Table 1, there were significant changes in the BBB in both groups. Neither inhibitors for ROS or PARP were able to prevent changes in the BBB integrity with both HCC1937 and HCC1937/WT although the changes were more dramatic with HCC1937 cells. Since COX-2 was more effective in vitro in preventing BBB permeability and TJ changes (Fig. 4C), we examined the potential of COX-2 inhibitor to inhibit BBB permeability changes and tumor colonization in brain. We employed 4T1-BrM5 mammary epithelial cells that express MT-BRCA1 and injected them into the mammary fat pads of BALB/c mice. Within 2 weeks, tumor growth in the mammary fat pads was observed as shown in Table 2A. COX-2 inhibitor inhibited tumor growth in vivo in the mammary fat pads (note different scale between panels A and B in Table 2). Further, COX-2 inhibitor decreased BBB permeability (10 mice per group per treatment), as analyzed by Evans blue test (data not shown). Further, in vivo imaging of the brain samples from these mice showed reduced colonization of 4T1-BrM5 tumor cells in brain by COX-2 inhibitor (1 out of 10 mice had tumor colonization in brain, Fig. 6B) as

compared to brain tumor colonization without treatment (10 out of 10 mice had tumor colonization in brain, Fig. 6A). Further, while tumor colonization of 4T1BrM5 was observed on day 10 (Fig. 6A) in Balb/c brain, we were able to observe tumor colonization in one mouse treated with COX-2 inhibitor only after 5 weeks (Fig. 6B).

Key research accomplishments:

1. ROS inhibitor or PARP inhibitor were not effective in vivo in preventing changes in the BBB integrity and colonization of tumor cells in brain.
2. WT-BRCA1 inhibited COX-2 expression in HCC1937 cells.
3. COX-2 inhibitor was more effective in inhibiting tumor growth in vivo, tumor colonization in brain, and in maintaining the BBB integrity.

Reportable outcomes:

A manuscript is in preparation.

Conclusion:

COX-2 inhibitors may be viable therapeutic approaches in addition to chemotherapy. In addition, they may minimize the risk for breast cancer metastasis to the brain in patients with mutated BRCA1.

Figure and table legends

Figure 1: BRCA1 mutant expressing cells induced changes in the distribution of ZO-1 and claudin-5 TJ proteins in HBMECs

3D cocultures of HBMECs with human astrocytes were either cocultured in the presence of HCC 1937 expressing MT BRCA1 (HCC MT) or wild type BRCA1 (HCC WT) for 6 hours and fixed immediately with 2% paraformaldehyde. Cells were then probed for ZO-1 and claudin-5 expression and visualized by indirect immunoflorescence using Alex Fluor 568 (red) conjugated secondary antibody (Molecular Probes). Nuclei were counterstained with DAPI (blue). Arrowheads indicated intact claudin-5 and ZO-1 TJs as indicated. Images shown are from a representative axial plane and are representative of over 30 fields examined in at least three independent experiments. Scale bar = 20 μm.

Figure 2: Effects of COX-2 inhibitor and ROS inhibitor of BMEC-TJs

HBMEC cocultures were preincubated with PARP inhibitor (30mM) or with ROS inhibitor (10mM selenium) for 6 hours. Immunostaining of TJs ZO-1 and claudin-5 was performed as described in Fig. 1.

Figure 3: Effects of ROS inhibitor on BMEC-TJs in the presence of HCC 1937 cells expressing mutant BRCA1 or WT BRCA1

HBMEC cocultures were pretreated with ROS inhibitor (50mM) for 1 hour. HCC-MT/BRCA1 or HCC-WT/BRCA1 cells were then added to HBMEC cocultures for 6 hours. The immunostaining of ZO-1 and claudin-5 TJs on BMECs were performed as described in Fig. 1.

Figure 4: Effects of HCC-BRCA1/WT and COX-2 inhibitor in vitro on BMEC-TJs

3D cocultures of HBMECs with human astrocytes were established and cocultured with HCC 1937 cells expressing WT BRCA1 in the absence of presence of COX-2 inhibitor (30 mM COX-

2 inhibitor celecoxib). TJs immunostaining of ZO-1 and claudin-5 were performed as detailed in Fig. 1.

Figure 5: Expression of COX-2 in HCC1937 and HCC1937/WT-BRCA1 by RT-PCR (Panel A) and Western blotting (Panel B)

A. For RT-PCR, we obtained mRNA from both cell lines. RNA concentration was 0.211 μg/μl for HCC1973 and 0.264 μg/μl for MCF-7. RT-PCR analysis was run with 158 bp primer (Zattelli et al.) at annealing temperature of 52℃ using Qiagen One step RT-PCR kit was used for the analysis.

B. Western blot analysis using COX-2 antibody from Santa Cruz (cat#sc-19999, lot F0510 product) with 72 kd.

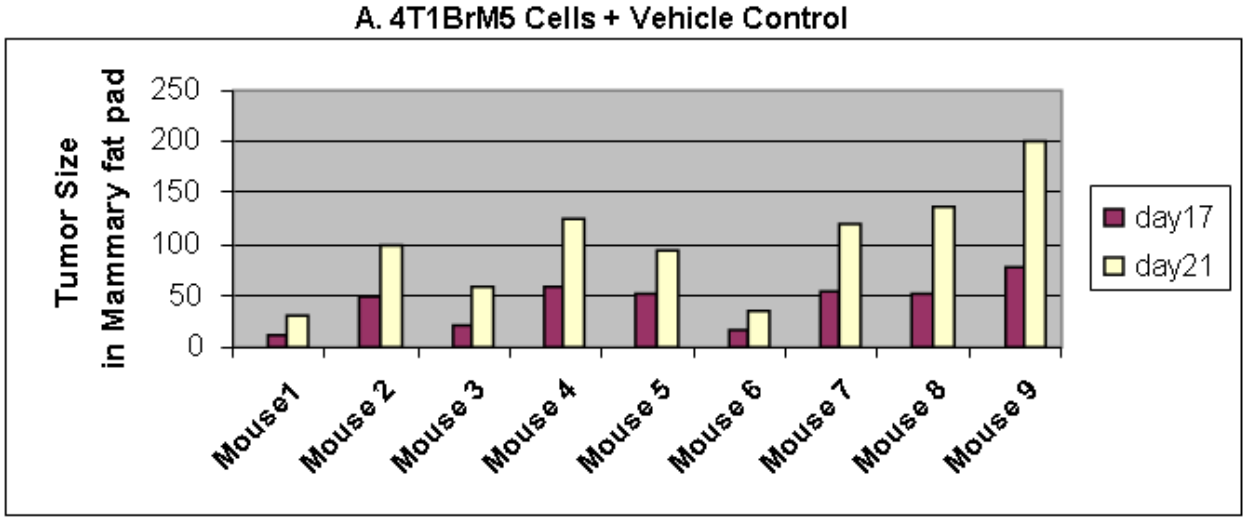
Figure 6: Internal images of murine mammary epithelial tumor (4T1BrM5-GFP) metastasis in brain

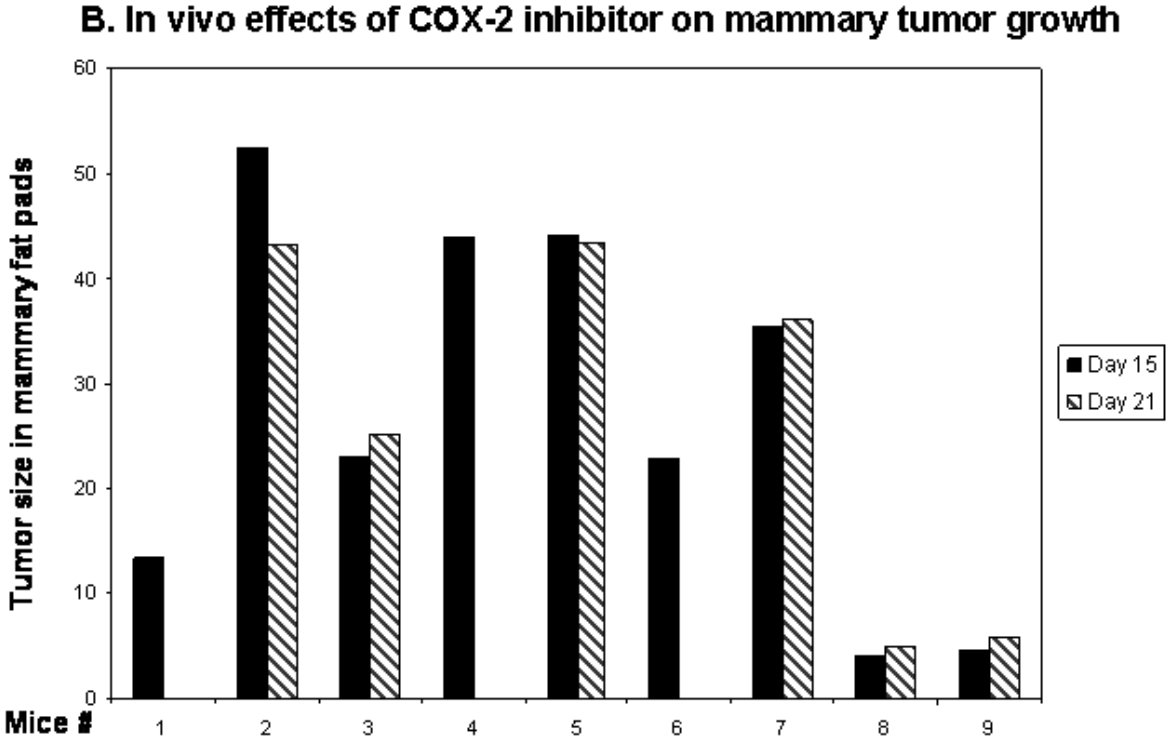
Murine breast metastasis in the mouse brain was imaged by GFP expression. Images show metastatic lesions in the brain.

A. Series of internal fluorescence images of metastatic lesions in the brain in mice administered with 4T1BrM5-GFP mammary tumor cells into mammary fat pads at day 10 or 17 as compared to WT control brain (C1, C2)

B. Series of internal fluorescence images of metastatic lesions in the brain in mice administered with 4T1BrM5-GFP cells and COX-2 inhibitor taken at week 5 following treatment. The GFP positive signal in the mammary tumor is shown as a positive control.

Table 2





BALB/c mice were administered either vehicle control (Panel A) or COX-2 inhibitor celecoxib at 10mg/kg (Panel B), one hour before injection of 4T1-BrM5 tumor cells (10^5 cells) into the mammary fat pads (10 mice per group per treatment). Live mice were then sacrificed at day 21 and tumor volume in the mammary fat pads was measured. Some of the mice died during the experiment. Therefore, we have no data for those mice, as indicated.

Figure 1

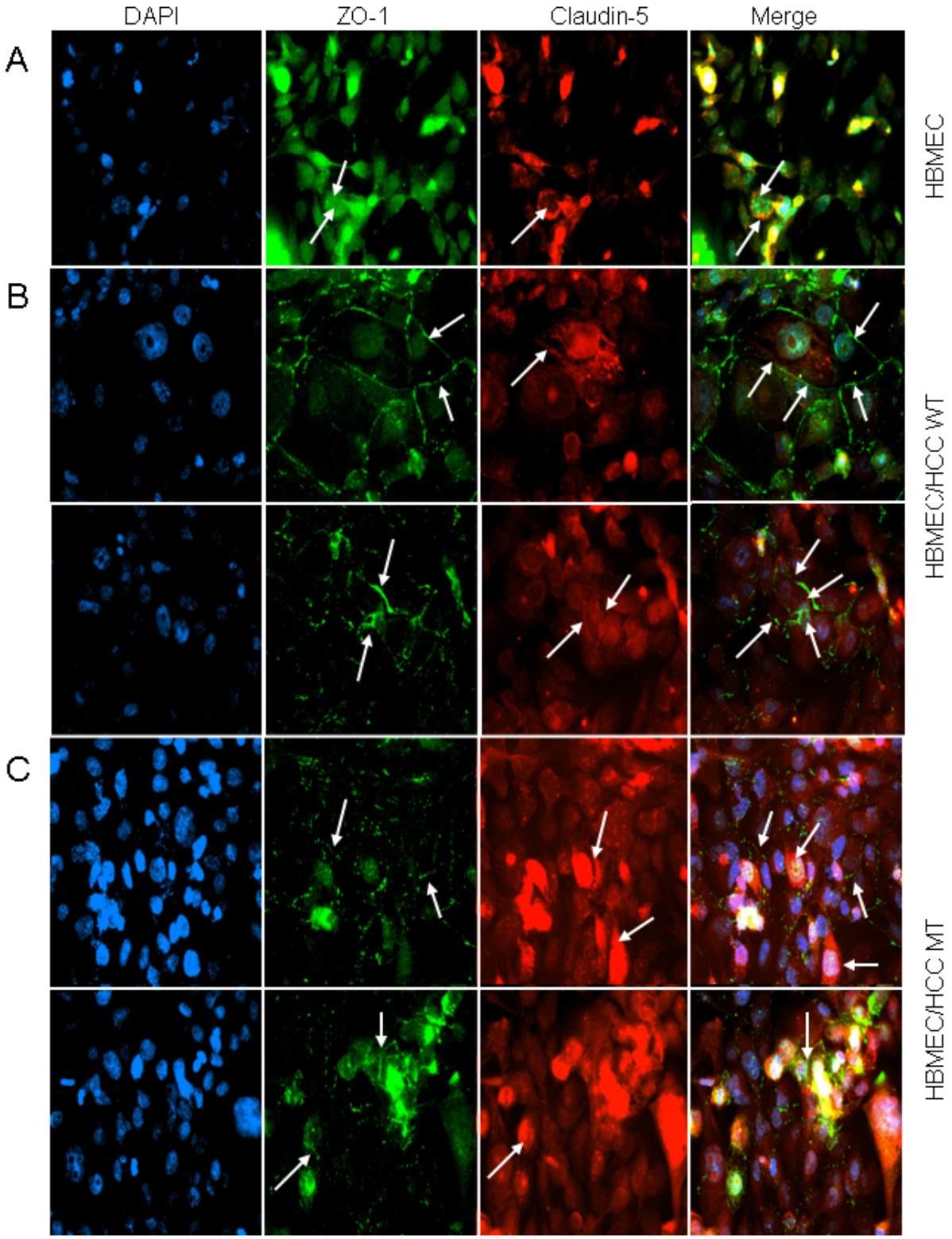


Figure 2

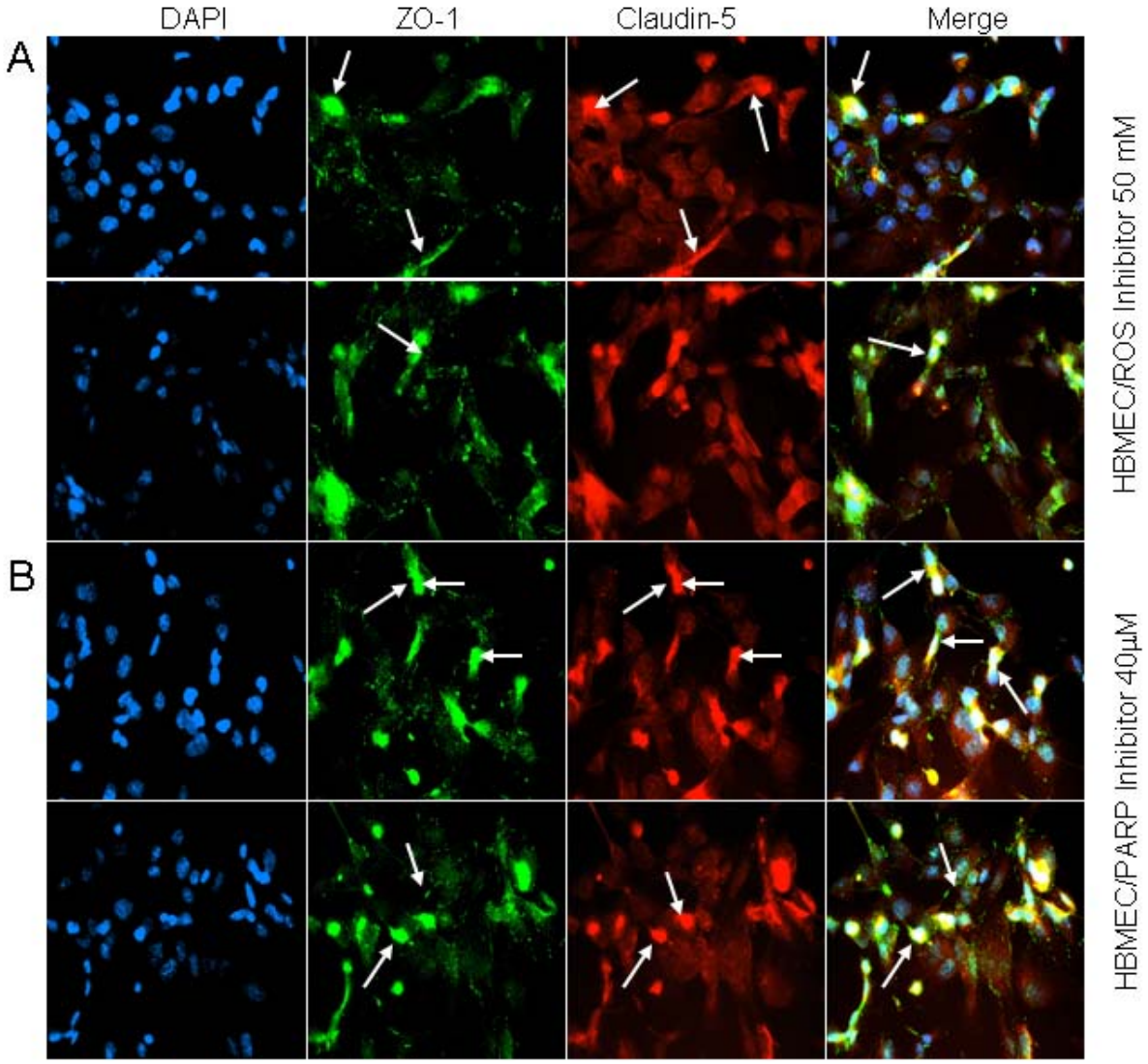

DAPI
ZO-1
Claudin-5
Merge
A
B
HBMEC/ROS Inhibitor 50 mM
HBMEC/PARP Inhibitor 40μM

Figure 3

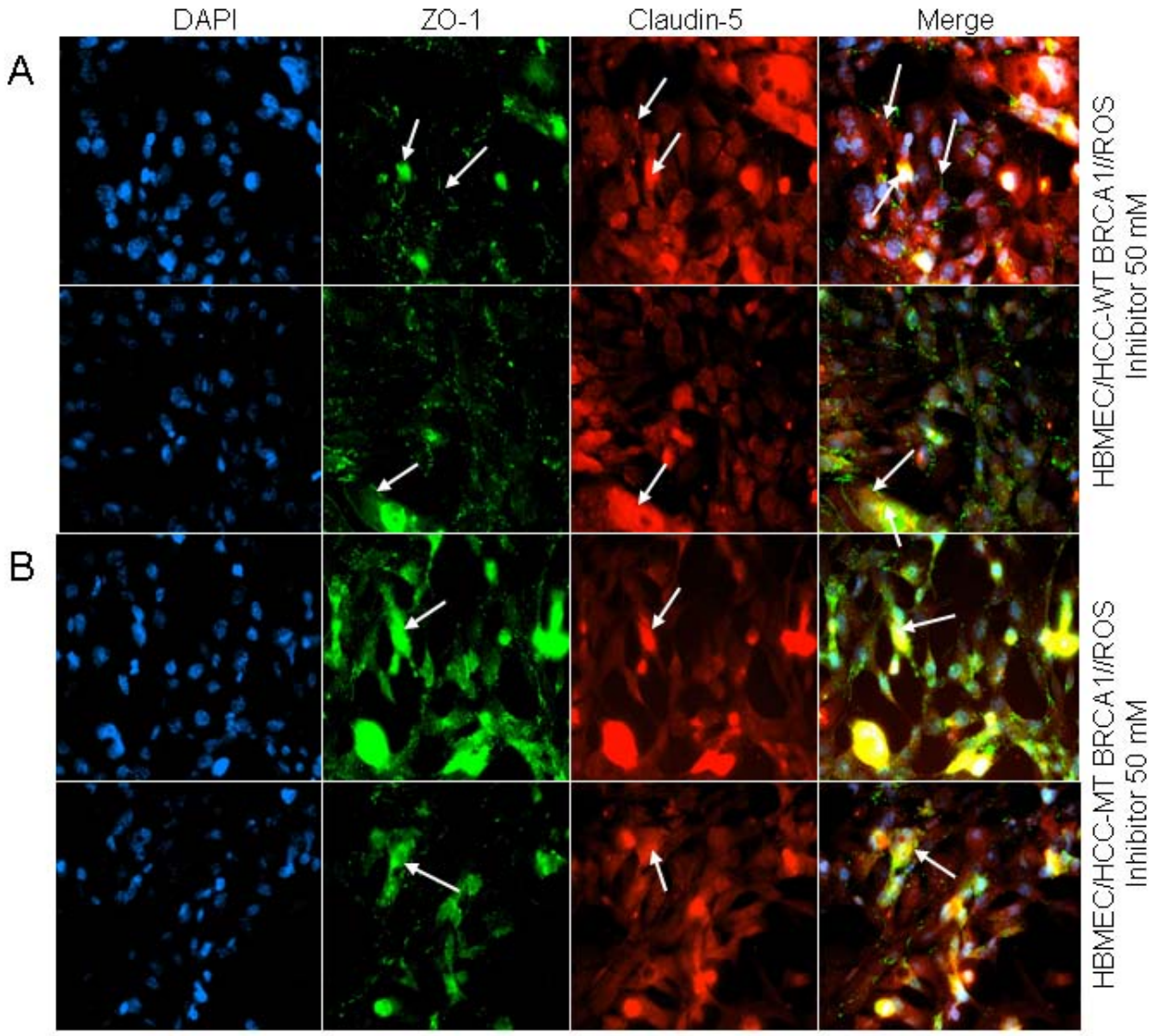
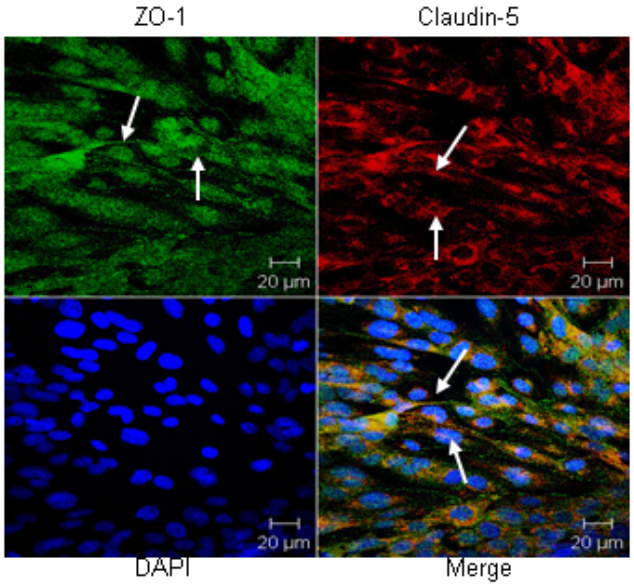
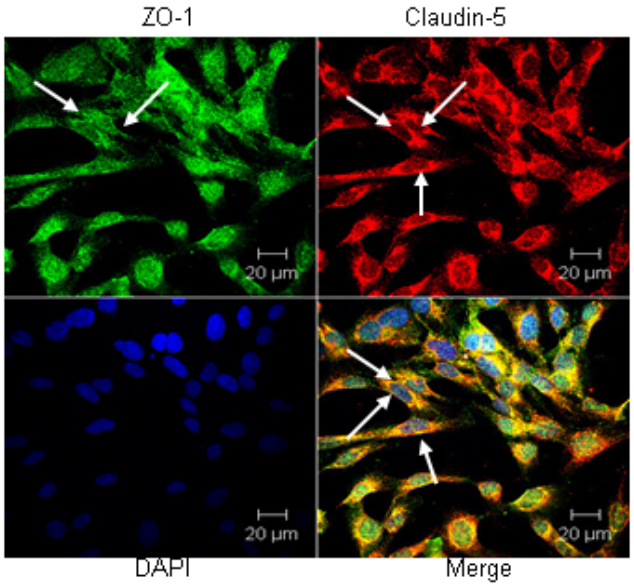

DAPI
ZO-1
Claudin-5
Merge
A
B
HBMEC/HCC-WT BRCA1//ROS
Inhibitor 50 mM
HBMEC/HCC-MT BRCA1//ROS
Inhibitor 50 mM

Figure 4

4A: HBMECs



4B: HBMEC / HCC-BRCA1 WT



4C: HBMEC / HCC-BRCA1 WT / COX-2 inhibitor

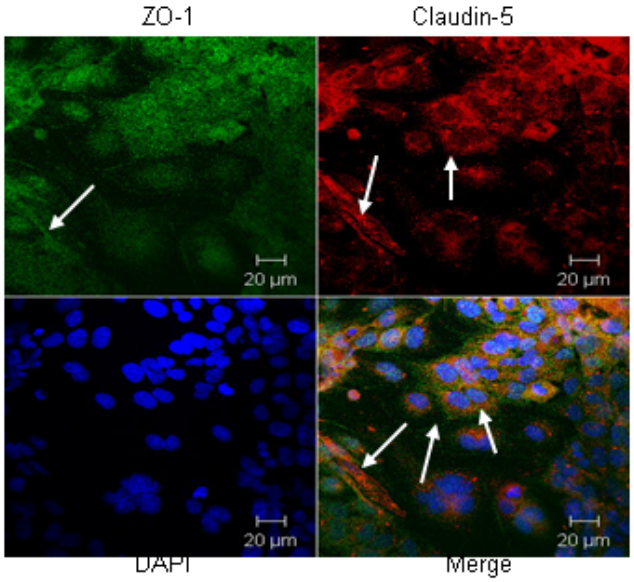
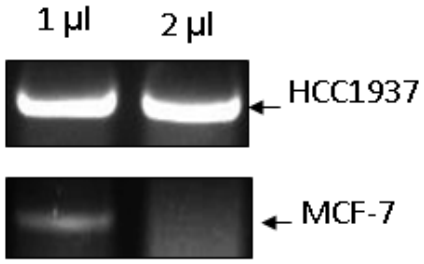


Figure 5

5A. COX-2 RT-PCR



5B. Western blot analysis of COX-2 expression in HCC1937/MT-BRCA1 and HCC1937/WT-BRCA1

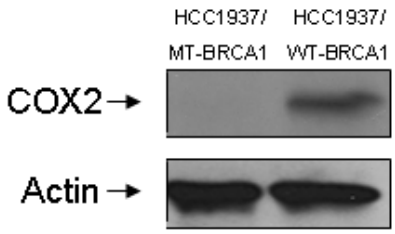
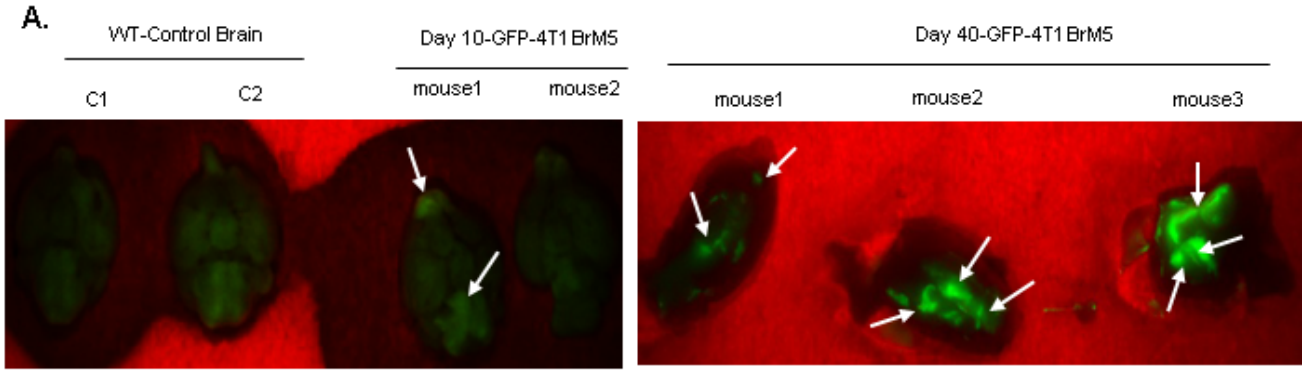


Figure 6


A.
WT-Control Brain
C1
C2
Day 10-GFP-4T1 BrM5
mouse1
mouse2
Day 40-GFP-4T1 BrM5
mouse1
mouse2
mouse3

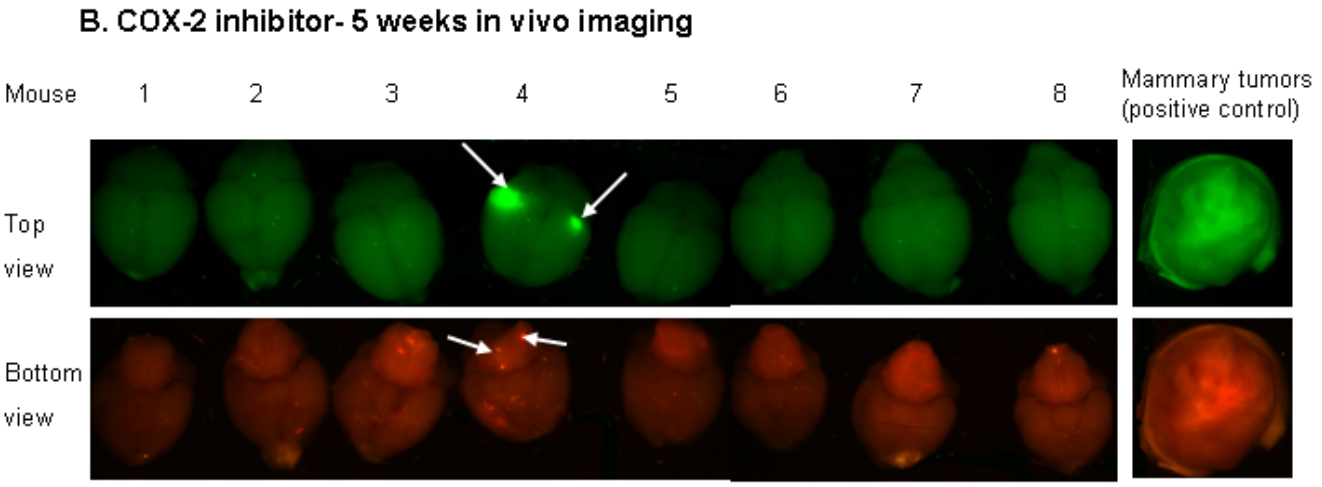

B. COX-2 inhibitor- 5 weeks in vivo imaging
Mouse
1
2
3
4
5
6
7
8
Mammary tumors (positive control)
Top view
Bottom view

Table 1

Treatment	Quantification of TJ change (%)	
	ZO-1	Claudin-5
WT-control brain	100%	100%
HCC/MT BRCA1 + vehicle control	42 ±8**	22 ±7*
HCC/MT BRCA1 + ROS inhibitor	37 ±11**	17 ±6*
HCC/MT BRCA1 + PARP inhibitor	41 ±14**	26 ±7*
HCC/WT BRCA1 + vehicle control	28 ±9**	21 ±11*
HCC/WT BRCA1 + ROS inhibitor	30 ±8**	20 ±7*
HCC/WT BRCA1 + PARP inhibitor	27 ±8**	19 ±8*

Quantitative analysis of TJs in vivo in brain: The changes is BBB TJs were analyzed 7 weeks following administration of HCC 1937/MT(HCC/MT)-BRCA1 or HCC 1937/WT(HCC/WT)-BRCA1 cells in the absence or presence of ROS inhibitor selenium (5mg/kg) or PARP inhibitor AZO228 (10mg/kg) i.v. one hour before tumor cell injection. Quantification of $CD31^+$ BMEC expression and TJ proteins claudin-5 and ZO-1 (n=12, random sections, 10 mice per group) was performed. The TJs of the samples were analyzed, quantitated and compared to control brain. The TJs in control brain were defined as 100%. Percent changes in treated samples, as compared with control brain were calculated. Data are presented as mean ±SD $^*p<0.05$, $^{**}p<0.005$.